Original Article

Itaconic Acid as A Differential Transcription Regulator of Apoptosis and Autophagy Pathways Genes: A Rat Adipose Mesenchymal Stem Cells Model

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Abstract

Objective: Itaconate, a novel regulatory immunometabolite, is synthesized by inflammatory macrophage. It acts as an anti-inflammatory mediator and regulates several metabolic and signaling pathways particularly Nrf2 pathway. The immunometabolites can affect the stemness potency, differentiation ability and viability of stem cells, but little is known about the critical function of Itaconate on the stem cell fate. The objective of the present study was to determine the regulatory effects of Itaconic acid on the cell viability and transcription of apoptosis and autophagy pathways genes in the rat adipose derived mesenchymal stem cells (ADMSCs).

Materials and Methods: In this experimental study, the ADMSCs were incubated with 125 μM and 250 μM dimethyl itaconate (DMI) for 24 hours or 48 hours. The expression of apoptosis pathway genes (*Bax, Bcl2, Caspase 3, Fas, Fadd* and *Caspase 8*) and autophagy pathway genes (*Atg12, Atg5, Beclin, Lc3b* and *P62*) were determined using real time polymerase chain reaction (PCR) assay. Using the ELISA method, cellular level of phospho-NRF2 protein was measured.

Results: The results indicated that DMI increased the expression of NRF2 protein, altered the expression of some apoptosis genes (*Fadd, Bax* and *Bcl2*), and changed the expression of some autophagy related genes (*Lc3b, Becline* and *P62*) in ADMSCs. DMI had no obvious effect on the transcription of caspases enzymes.

Conclusion: Because autophagy activation and apoptosis suppression can protect stem cells against environmental stress, it seems Itaconate can affect the functions and viability of ADMSCs via converse regulation of these pathways.

Keywords: Adipose Derived Mesenchymal Stem Cells, Apoptosis, Autophagy, Itaconate

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Introduction

Treatment application of mesenchymal stem cells (MSCs) for various diseases has been remarkably increased. The adipose derived MSCs (ADMSCs) are a type of adult stem cells with unique ability of proliferation, differentiation and immuno-modulation. ADMSCs have been regarded as excellent sources for cell therapy, tissue regeneration and autologous transplantation (1). ADMSCs are one of the best sources of MSCs due to simple isolation and abundant in number (2).

Recent studies have revealed a complex interaction between the inflammatory cells and MSCs. Interaction between MSCs and the inflammatory environment plays an important role in the regulating immune responses against transplanted cells. Although, the interaction between MSCs and immune cells have been confirmed in previous studies, there is little insight into the specific effect of the inflammatory immunometabolites on the MSCs fate. However, this molecular interaction is one main future area of research (3). MSCs have a dual effect on the inflammation process; depending on the environmental conditions they can intensify or alleviate the inflammation process (4, 5). MSCs regulate the function of innate and adaptive immune systems by affecting the inflammatory microenvironment. The immune factors secreted by inflammatory cells have various impacts on the MSCs phenotype such as stemness potency, differentiation ability, viability and efficiency of transplantation (3, 6). Several studies have demonstrated that long-term culture of MSCs can alter the proliferation potency and vital activity of MSCs by affecting the autophagy and apoptosis pathways (7). Autophagy and apoptosis are two evolutionarily conserved processes that play a crucial role in the stem cell fate determination. Apoptosis or programmed cell death is induced by death receptor-dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is activated by TNF family ligands, while the intrinsic pathway is activated by different factors such as UV irradiation, chemotherapy, growth factor withdrawal

or cytokine deprivation (8). Autophagy contributes to the routine turnover of cytoplasmic components, and as part of tissue homeostasis. Different protein complexes control the formation and activation of the autophagosome. *Atg15*, *Atg5* and *Lc3b-II* genes are responsible for the formation of mature autophagosome. The P62 is an important autophagy protein that delivers ubiquitinated proteins to the proteasome for degradation and facilitates nuclear and cytosolic protein quality control (9). Autophagy can regulate different cellular processes in the stem cells, including self-renewal, differentiation, senescence, and apoptosis. Expression of several cellular proteins, including transcription factors, adhesion molecules and secreted proteins which are essential for self -renewal and stem cell differentiation are controlled by autophagy pathway (8, 9). It has been reported that autophagy helps cells survive during prolonged starvation and other microenvironment stresses. Recent studies have indicated that autophagy can act either as an inducer or as a suppressor of differentiation and apoptosis process in the MSCs (10, 11). Several effects of inflammatory factors on the MSCs are mediated through the regulation of apoptosis and autophagy pathways. Dang et al. (12) demonstrated that sepsis-induced inflammation stimulates autophagy and apoptosis in MSC. Generally, up-regulation of autophagy-related genes following oxidative stress and nutritional deprivation help MSCs to increase their survival. Following autophagy activation, defective mitochondria are removed by the autophagosomes and the production of free radicals that stimulate the inflammatory cytokine production are suppressed (9).

Previous studies have shown that immune cells produced several inflammatory mediators that affect stem cell functions and fate (3). Macrophages, a critical cell of the immune system can affect the cellular homeostasis in response to different microenvironmental cues. Recently, the complex cross-talk between MSCs functions and macrophages has been reported. MSCs affect macrophage polarization by secreting various factors such as IL-6, prostaglandin E2 (PGE2) and exosome (13). Recent studies have also shown that macrophages can also affect MSCs viability, proliferation and differentiation by secretion of various immune factors, indicating a critical interaction between MSCs and macrophages under the physiological condition (14).

Since anti-inflammatory effect, Itaconate, an immunometabolite, has attracted much attention. Itaconate is synthesized in response to LPS and certain infections by macrophages. The immune-responsive gene 1 (*Irg-1*) is expressed in mammalian macrophages under inflammatory conditions and encodes the mitochondrial enzyme cis-aconitic acid decarboxylase (CAD). CAD in turn catalyzes the cis-aconitic acid decarboxylation to produce itaconate during the tricarboxylic acid (TCA) cycle (15, 16). The process of itaconate production is also activated in myeloid cells under inflammatory conditions (17, 18). Recent published studies indicated that itaconate and its cell permeable derivatives, dimethyl itaconate (DMI) and 4-octyl itaconate (OI) markedly suppress the production of pro-inflammatory mediators in the lipopolysaccharide-treated macrophages and inflammatory associated diseases (19). Several molecular mechanisms have been reported for anti-inflammatory actions of itaconate in macrophage, including (node like receptor family pyrin domain containing 3 (NLRP3) inflammasome suppression, mitochondrial reactive oxygen species (ROS) production inhibition, NFκβ inflammatory pathway inactivation and nuclear factor erythroid 2-related factor 2 (NRF2) activation (20, 21). Previous studies have shown the anti-inflammatory actions of itaconate in the immune cells, to our knowledge, no study has yet been conducted to detect the effect of itaconate or its derivatives on the MSCs functions and fate. Therefore, the present study was designed to identify the effect of DMI on the vital activity and expression of apoptosis and autophagy related genes in the rat ADMSCs.

Materials and Methods

All animal work was carried out with approval from the Ethics Committee for research in animals and humans of Shahid Chamran University of Ahvaz (ee/97.24.03.93442/scu.ac.ir). Working with animals was also carried out on the basis of the guideline for the care and use of laboratory animals (NIH publication no. 86-23).

Animals

In this experimental study, six Sprague Dawley rats (8 weeks old, ~180 g) were obtained from the center of laboratory animals of the faculty of veterinary medicine of Shahid Chamran University of Ahvaz, Khozestan, Iran. They were kept under our animal facilities (22 ± 1℃), with a 12-hours light: 12-hours dark cycle beginning at 7:00 a.m. During experiments, the animals had free access to water and rat pellet diet (Pars, Tehran, Iran). Animals were euthanized with a combination of ketamine hydrochloride (Alfasan, Nederland) and xylazine (Alfasan, Nederland) (100 mg/kg of Ketamine and 10 mg/kg of Xylazine).

Isolation and culture of ADMSCs

Adipose tissue was surgically attained from the inguinal and epididymal fat pads and sliced into small pieces. The adipose tissues were digested with DMEM-HG medium (BI-1001, Bioidea, Iran) containing 0.1% collagenase type 1 (C0130, Sigma-Aldrich, USA) and 1% penicillin/streptomycin (BI-1230, Bioidea, Iran) for 40 minutes at 37°C in a shaking bath. Collagenase was neutralized by adding DMEM-HG containing 10% fetal bovine serum (FBS, BI-1201, Bioidea, Iran) and centrifuged at 1200 × rpm for 7 minutes to obtain a pellet. The supernatant was removed and precipitated cells were re-suspended in the DMEM-

HG medium containing 15% FBS and1% penicillin/streptomycin (BI-1230, Bioidea, Iran) and seeded in 25 cm^2 culture flask and incubated at 37˚ C with 5% CO_2. The residual non-adherent red blood cells were removed by washing with the DMEM-HG medium. The medium was changed every 3 days and after the cells reached the third passage, the cells were passaged using 0.25% trypsin-EDTA (BI-1602, Bioidea, Iran). The cells in the third passage were used for the next steps of the experiment.

Identification of ADMSCs phenotype

To confirm the phenotype of ADMSCs, the expression of CD31, CD44, CD45 and CD90 surface markers was determined using the flow cytometry method as described previously. To determine the lineage differentiation potency of ADMSCs, the cells were cultured in osteogenic and adipogenic differentiation medium for 14 days as described previously. Then the cells were fixed with 4% Paraformaldehyde (158127, Sigma-Aldrich, USA) for 10 minutes and stained with 40 m*M* Alizarin Red (58005, Merck, USA) or 0.5% Oil Red O (23125, Merck, USA) to identify the calcium deposition and fat droplet formation (22).

Treatment of ADMSCs with itaconate

The third passage of ADMSCs was seeded at a density of 1.5×10^5 cells/ml in the DMEM-LG medium containing 125 µM and 250 µM of DMI for 24 hours or 48 hours The culture media were supplemented with 5 % FBS and 1% pen/strep antibiotics. Compounds were prepared as 125 µM and 250 µM stock solutions in cell media and diluted directly into the culture media. DMI was prepared directly in the DMEM-HG cell media in order to avoid solvent effects. Control cells were cultured in the absence of DMI for the indicated times. At the end of experimental periods, the cells were detached using 0.25% Trypsin-EDTA (BI-1602, Bioidea, Iran) and used for subsequent experiments. We performed all experiments using 125 uM and 250 uM of DMI a dose shown to reduce LPS-induced inflammation in previous studies (17, 23).

Effect of DMI on cell proliferation

A sulforhodamine B (SRB) assay was performed to examine cell proliferation. Briefly, ADMSCs were seeded in 96-well plates in DMEM-LG (BI-1002, Bioidea, Iran) growth medium at a density of 5000 cells/well, respectively and settled overnight at 37˚C with 5% CO_2. The cells were then treated with 125 uM and 250 uM of DMI. At days 1, 3, 5 and 7 after DMI treatment, cells were fixed with cold 10% (w/v) Trichloroacetic acid (TCA) (T6399, Merck, Germany). The plates were washed with water, stained with 0.4% SRB (w/v, dissolved in 1% acetic acid) (10056, Merck, USA) and washed with 1% acetic acid (10056, Merck, USA). The protein-bound dye was subsequently dissolved in 10 mmol/L Tris-HCl (252859, Sigma Aldrich, USA). Absorbance values at 540 nm were recorded using a colorimetric plate reader (SP2, BioTek, USA) (24).

RNA extraction and cDNA synthesis

The RNAs were extracted using the RNX isolation reagent (EX6101, SinaClon, Iran) according to manufacturer's protocol. The samples were treated with DNase I enzyme (mo5401, SinaClon, Iran) to avoid DNA contamination. The quality and concentration of the extracted RNAs were assessed using a NanoDrop (2000c, Thermo Scientific, USA). High pure RNA, *OD* 260/280 ratios ≥1.8, was used for cDNA synthesis. The cDNA was synthesized using YTA cDNA synthesis kit (YT4500, Yekta Tajhiz, Iran) and random hexamer primer (YT4550, Yekta Tajhiz, Iran) by using 0.5 µg of RNA as recommended by the manufacturer.

Real time polymerase chain reaction assay

Real-time polymerase chain reaction (PCR) was performed using the StepOnePlus™ Real-Time PCR System (StepOnePlus™, Applied Biosystems, USA) by the qPCR™ Green Master Kit for SYBR Green I® (YT2551, Yektatajhiz, Iran). The primers were designed using the Primer3 software version 4.1.1 (Table 1). Relative expression levels of the target genes were compared to rat *Gapdh* as a housekeeping gene. The thermal program was consisted of 95˚C for 5 minutes, followed by 40 cycles of 94˚C for 15 seconds, 60˚C for 15 seconds and 72˚C for 30 seconds. All experiments were performed in triplicate. Two separate reactions without cDNA or with RNA were performed in parallel as controls. The results were analyzed by comparative $2^{-\Delta\Delta Ct}$ method using Lightcycler 96® software.

Determination of nuclear NRF2 protein

The nuclear NRF2 protein concentration was measured using Nuclear/Cytosol Fractionation Kit (K266, Biovision, USA) as recommended by the manufacturer. Protein concentration of the nuclear fraction was estimated using the Bradford method. NRF2 concentration was determined using rat specific NWLSS™ NRF2 ELISA kit (NWK-NRF2H, Northwest Life Science Specialties, Canada) and the concentration was expressed as ng/10^6 cells. All experiments were performed in duplicates.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism8 software (GraphPad Software, Inc., San Diego, CA). All data were presented as mean ± standard deviation (SD). The normality of data or equality of error variances was determined using Shapiro–Wilk or Levene's tests. Two-way analysis of variance (ANOVA) was used to determine the interactions of sampling times and DMI concentration on each factor. Mean values were compared between different treated groups at different time points using one-way ANOVA and Tukey multiple-comparison post hoc tests. A statistically significant difference between different experimental groups at each sampling time was represented as follows: *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Table 1: Characteristics of primers that used in the present study

Gene name	Primer sequences (5´-3´)	Size (bp)	GenBank accesion No
Gapdh	F: AGTTCAACGGCACAGTCAAG R: TACTCAGCACCAGCATCACC	119	XM_017593963.1
Caspase 3	F: CTATCCATGGAAGCAAGTCGATG R: TTGCGAGCTGACATTCCAGT	136	NM_012922.2
Bcl2	F: ATCGCTCTGTGGATGACTGAGTAC R: AGAGACAGCCAGGAGAAATCAAAC	135	NM_016993.2
Bax	F: ATCGCTCTGTGGATGACTGAGTAC R: AGAGACAGCCAGGAGAAATCAAAC	144	NM_017059.2
FADD	F: AGGGATCTGTGAGCAAGAGT R: GGCACTTGGTGCTACATCAT	143	AJ441127.1
FASL	F: AGCACACCCTCTGAAACCAA R: ATACGAAGTACAACCCAGCCTC	172	NM_012908.1
Caspase 8	F: AGGTTTCTGCCTACAGGGTT R: GCTCGAGTTGTCTTGCAGTT	125	XM_039084164.1
P62	F: CAGCTGCTGTCCGTAGAAATTG R: ACCCGCTCTTTCAGCTTCAT	113	NM_130405.2
Beclin1	F: TCAGGAACTCACAGCTCCATT R: ACCATCCTGGCGAGTTTCAA	112	NM_053739.2
Atg5	F: GAGAAGCAGAGCCATACTATTTGC R: TTTCAGGGGTGTGCCTTCAT	146	NM_001014250.1
Atg12	F: TGTCCAAGCACTCATCGACTT R: CCATCACTGCCAAAACACTCAT	141	NM_001038495.1
LC3	F: GACAGCACTGGCTGTTACAT R: AGCAGAGGCTTGCTTTAGTTG	109	XM_017601351.1

Results

Characterization of isolated ADMSC

To determine the phenotype of isolated ADMSCs, we determine their morphology, surface markers expression and differentiation potency. Flow cytometry analysis revealed ADMCs were positive for expression of cluster of differentiation (CD) CD90 and CD105, but negative for CD31 and CD45 (Fig.1A). In passage 3, ADMCs exhibited a spindle-shaped morphology (Fig.1B1). To investigate the differentiation capacity of isolated ADMSCs, cells were cultured in the osteogenic and adipogenic differentiating media and lineage potential was tested by staining for the typical lineage markers. Osteogenesis was determined by a bone-type marker, Alizian red staining (Fig.1B2) and adipogenesis was stained with oil Red O, cytoplasmic lipid droplets (Fig.1B3). These results confirmed that isolated ADMSCs successfully differentiate into the multiple cell types including osteoblasts and adipocytes.

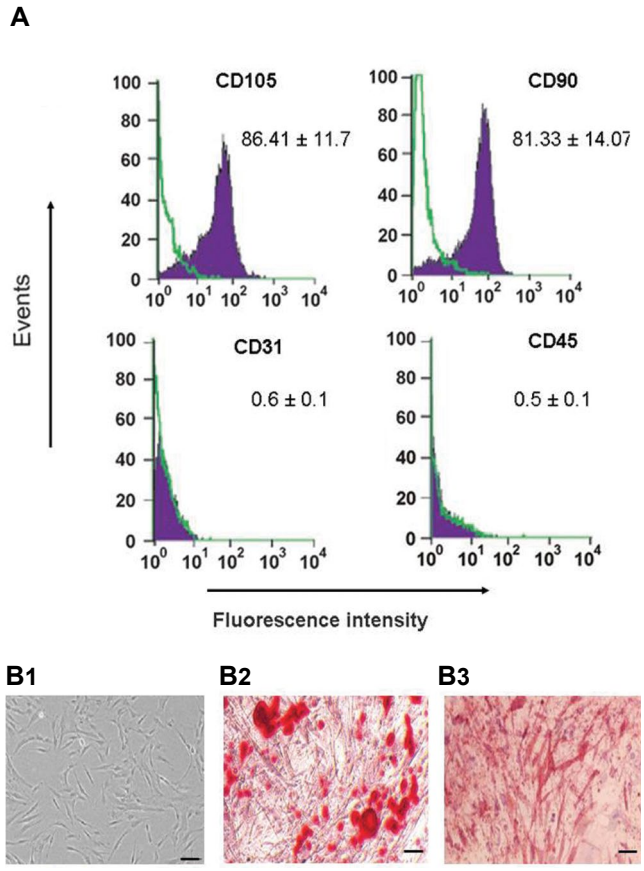

Fig.1: Characterization of adipose derived mesenchymal stem cells (ADMSCs) isolated from epididymal and inguinal adipose fat pads of rats. **A.** Flow cytometer analysis revealed the positive expression of CD105 and CD90 and negative expression of CD31 and CD45 in the ADMSCs. **B1.** Morphology of cultured ADMSCs. ADMSCs exhibited spindle-shaped morphology. **B2.** Osteogenic differentiation was confirmed by Alizian red staining. **B3.** Adipogenesis was detected by accumulated lipid vacuoles within the cytoplasm that stained with oil red O (scale bar: 50 µm, magnification: 100×). Numbers on graphs represents the percentage of cells that express each cell surface marker.

Effect of DMI on cell proliferation and nuclear Nrf2 concentration

We observed that ADMSCs exposure to a dose of 125 uM DMI resulted in higher cell density at days 3, 5 and 7 after treatment in comparison with the untreated cells (P<0.05). ADMSCs that treated with 250 µM DMI showed higher cell density at day 3, 5 and 7 after exposure in comparison with the untreated cells and cells treated with another dose of DMI (P<0.01, P<0.001, Fig.2A). These findings indicated that DMI could increase the cell proliferation in a dose dependent manner in MSCs.

Because it has been previously found that DMI exerts its effects in other cells through the NRF2 pathway, we determined the nuclear levels of NRF2 in DMI treated ADMSCs to determine the activation of the NRF2 pathway by DMI in ADMSCs. Our results showed that DMI in a dose and time dependent manner could increase the nuclear level of NRF2 protein in ADMSCs in comparison with untreated cells. DMI at a dose of 250 µM for 48 hours. had the maximal stimulatory effect on the translocation of NRF2 protein to nucleus compared to other dose and incubation time (P<0.0001, P<0.001, Fig.2B).

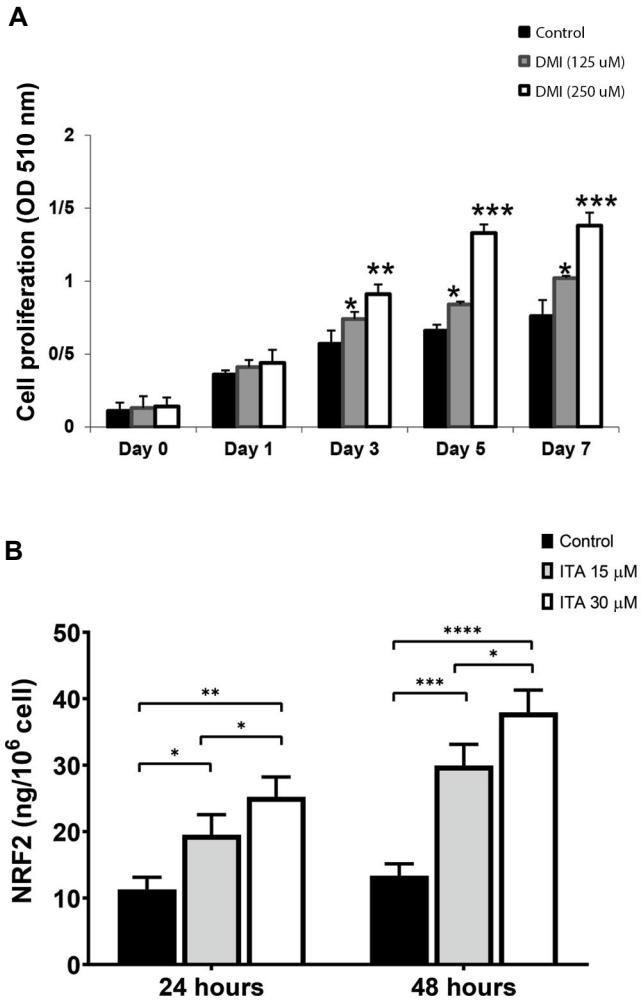

Fig.2: Evaluation of cell proliferation and cellular NRF2 protein levels in the dimethyl itaconate (DMI) treated adipose derived mesenchymal stem cells (ADMSCs). **A.** Cell proliferation assay using Sulforhodamine B method. ADMSCs proliferation was assessed up to 7 days, in the presence of 125 µM and 250 µM of DMI. **B.** The nuclear concentration of NRF2 protein in the ATDMCs following different treatment. For each concentration, the mean ± SD for at least three independent experiments is represented. *, **, ***, **** represent the significant difference between DMI treated groups and the control group at P<0.05, P<0.01, P<0.001 and P<0.0001, respectively.

Effect of DMI on transcription of external apoptosis pathway genes

Treatment of ADMSCs with both concentrations of DMI significantly reduced the *Fadd* expression after 24 hours and 48 hours exposure in comparison with the untreated group (P<0.01, P<0.001). Down regulation of the *Fadd* transcription level after exposure to 250 µM DMI at 48 hours was more than 24 hours time period (P<0.01, Fig.3A). Treatment of ADMSCs with both concentrations of DMI at 24 hours and 48 hours had no significant effect on the genes expression, *Fasl* and *Caspase 8* (Fig.3B, C).

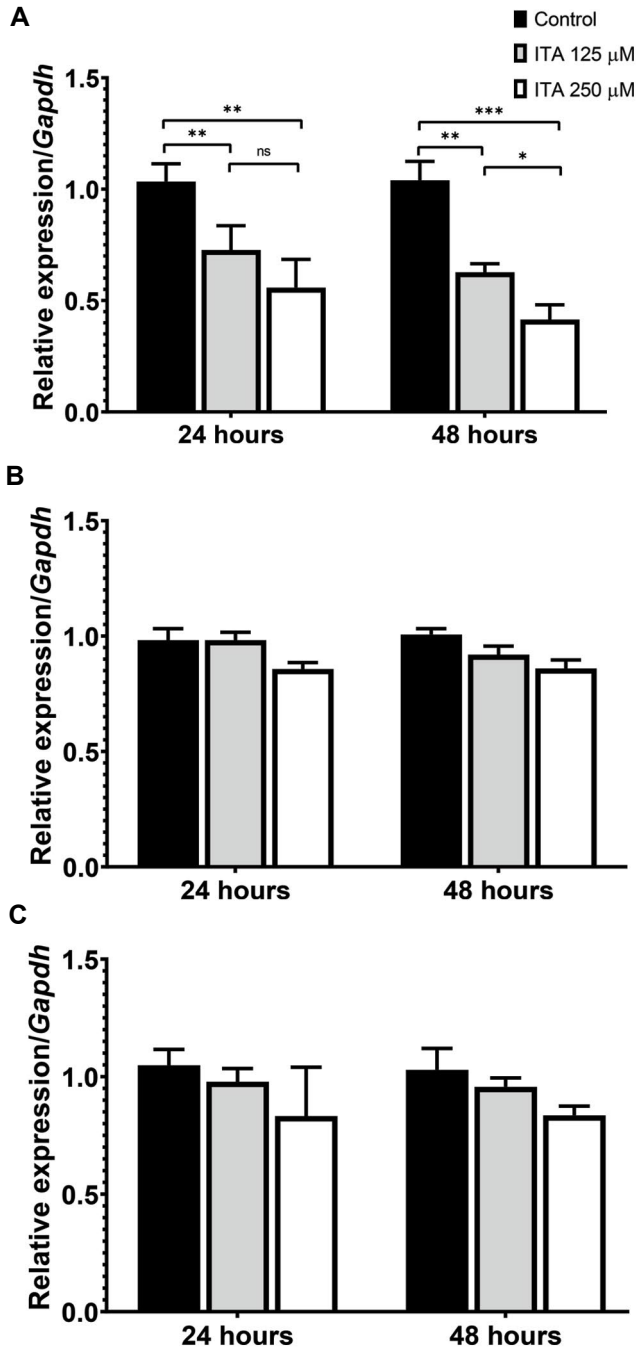

Fig.3: The mRNA levels of *Fadd*, *Fasl* and *Caspase 8* in adipose derived mesenchymal stem cells (ADMSCs) following different treatment. **A.** *Fadd*, **B.** *Fasl*, and **C.** *Caspase 8. Gapdh* was used as a housekeeping gene. *, **, *** represent the significant difference between dimethyl itaconate (DMI) treated groups and the control group at P<0.05, P<0.01, and P<0.001, respectively. ns; Non significant.

Effect of DMI on transcription of internal apoptosis pathway genes

Our results revealed that exposure of ADMSCs with the 125 μM DMI for 24 hours or 48 hours had no significant effect on the transcription level of the *Bax* gene (Fig.4A). DMI at a dose of 250 uM caused a downregulation of the *Bax* gene in the ADMSCs at both incubation times in comparison with the untreated cells (P<0.01, P<0.001) and down regulation of *Bax* transcription level in 48 hours was more than 24 hours time period (P<0.01, Fig.4A). ADMSCs cells that treated with the 250 μM DMI for 24 hours or 48 hours showed a significant increase in the mRNA level of the antiapoptotic *Bcl2* gene in comparison with the untreated cells (P<0.001, P<0.0001, Fig.4B). Treatment of ADMSCs cells with the 125 μM DMI for 24 hours had no significant effect on the transcription level of the *Bcl2* gene (P<0.01, Fig.4B). The highest upregulation of the *Bcl2* transcription level was observed in the ADMSCs after exposure to the 250 μM DMI for a 48 hours time period (P<0.0001, Fig.4B). Our results showed that treatment of ADMSCs with both doses of DMI in the different time periods had no significant effect on the *Caspase 3* expression level (Fig.4C).

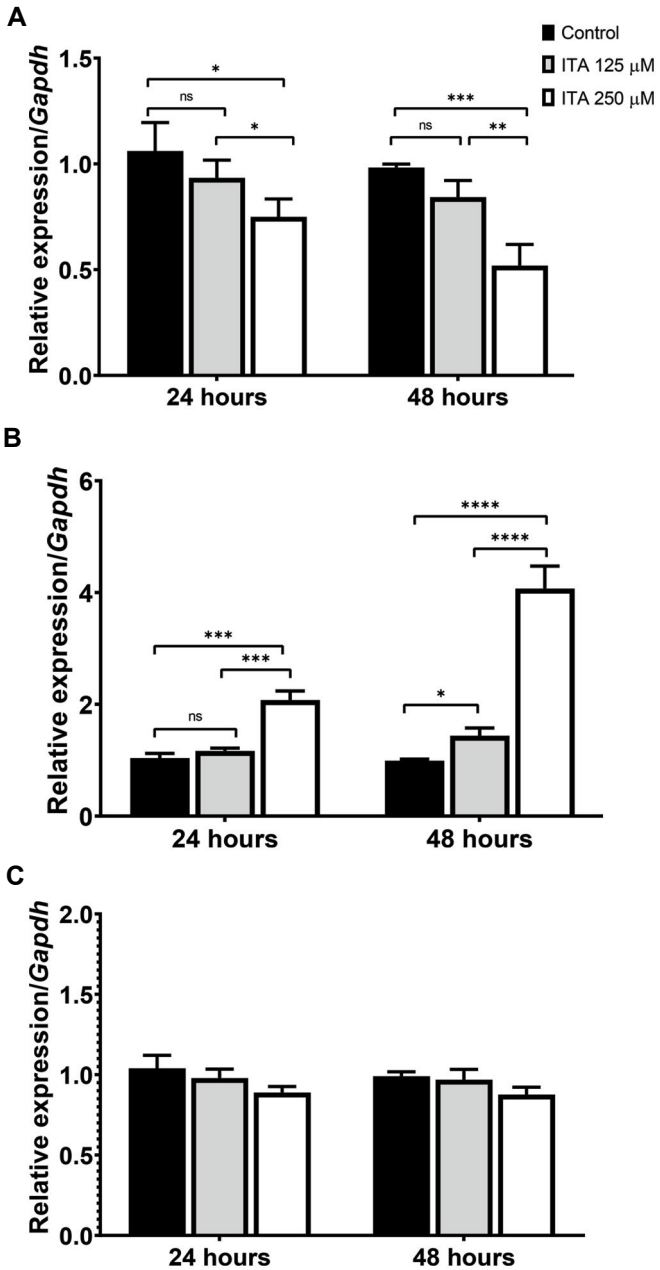

Fig.4: The mRNA levels of *Bax*, *Bcl2* and *Caspase 3* in the adipose derived mesenchymal stem cells (ADMSCs) following different treatment. **A.** *Bax,* ***B.*** *Bcl2*, and **C.** *Caspase 3*. *Gapdh* was used as a housekeeping gene. *, **, ***, **** represent the significant difference between dimethyl itaconate (DMI) treated groups and the control group at both time periods at P<0.05, P<0.01, P<0.001, and P<0.0001, respectively.

Effect of DMI on transcription of autophagy associated genes

qRT-PCR analysis revealed that DMI at both doses for 24 hours and 48 hours time period could stimulate the transcription of the *Lc3b* gene in ADMSCs in comparison with the untreated cells (P<0.01, P<0.001, Fig.5A). DMI at a dose of 250 µM at 48 hours incubation time had the maximum stimulatory effect on the expression level of *Lc3b* in comparison with other doses and incubation time (P<0.0001, Fig.5A). Following treatment with DMI at a dose of 125 µM and 250 µM for 24 hours or 48 hours the expression level of *Beclin* gene was increased in comparison with the untreated cells (P<0.0001). High and low concentrations of DMI at both incubation time had a similar stimulatory effect on the expression level of the *Beciln* gene in ADMSCs *(*Fig.5B). Exposure of ADMSCs to 125 µM DMI for 24 hours had no obvious effect on the transcription level of the *P62* gene, while a 48 hours exposure of ADMSCs to this dose could significantly induce the transcription level of the *P62* gene in comparison with the untreated cells (P<0.01). DMI at a dose of 250 µM had a more stimulatory effect on the expression level of the *P62* gene in comparison with another dose of DMI at both exposure times (P<0.01, P<0.05, Fig.5C). DMI at both concentrations for 24 hours and 48 hours had no significant effect on the mRNA levels of *Atg5* and *Atg12* genes in the ADMSCs in comparison with the untreated cells (Fig.5D, E).

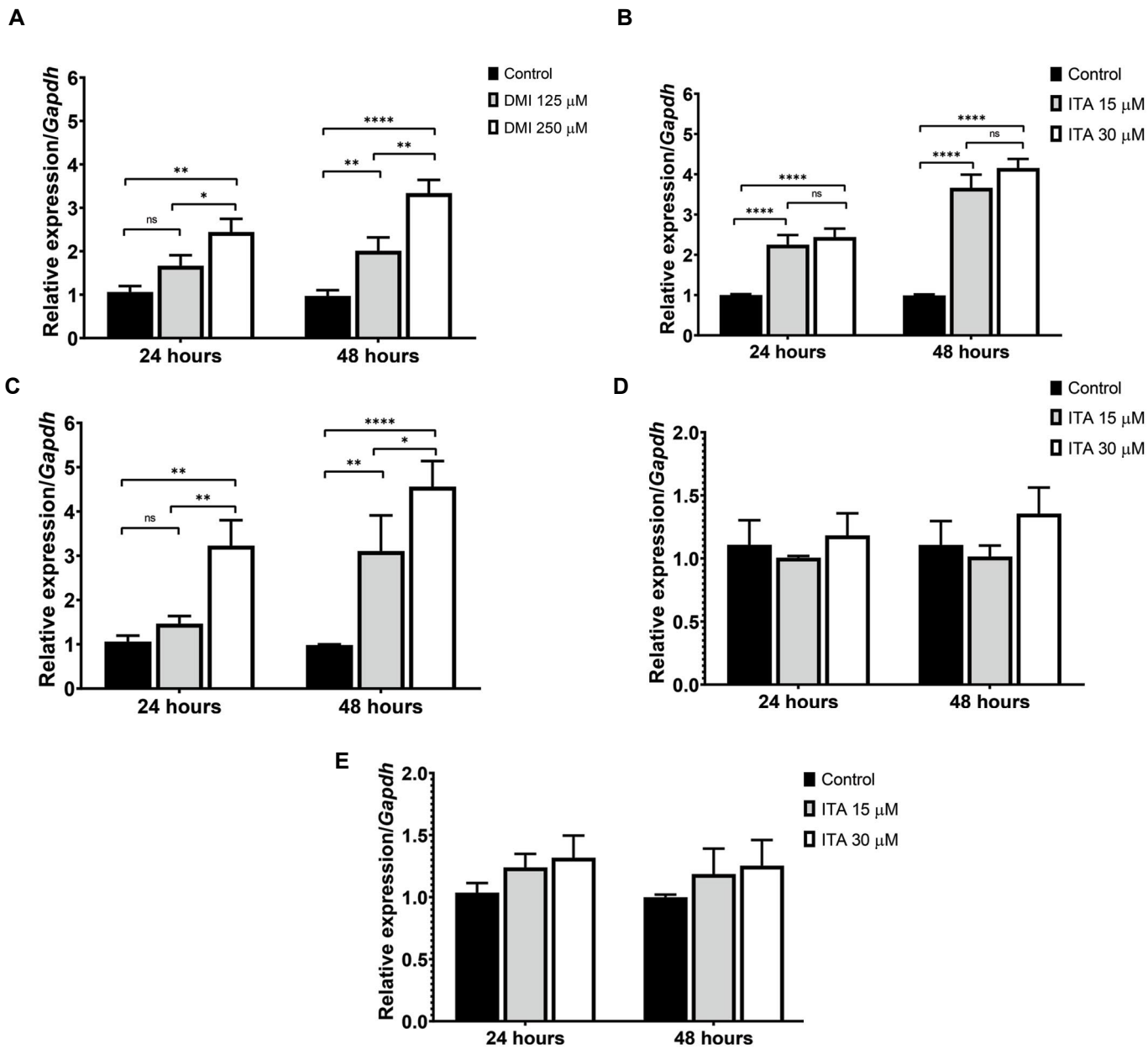

Fig.5: The mRNA levels of *L3b, Belin, P62, Atg5,* and *Atg12* in the adipose derived mesenchymal stem cells (ADMSCs) treated with different concentrations (125 µM and 250 µM) of dimethyl itaconate (DMI) at different exposure time (24 hours and 48 hours). **A.** *Lc3b*, **B.** *Beclin*, **C.** *P62*, **D.** *Atg5*, and **E.** *Atg12*. Gapdh was used as a housekeeping gene. *, **, ***, **** represent a significant difference between the DMI treated groups and the control group at both time periods at P<0.05, P<0.01, P<0.001, and P<0.0001, respectively.

Discussion

Itaconate is a novel anti-inflammatory mediator which is produced by mammalian immune cells (19). The role of itaconate as a novel immunometabolite on the stem cell fate in physiological condition is still not well understood. The present study investigated the effect of DMI, a membrane permeable non-ionic form of itaconate, on the vital activity and transcription level of apoptosis and autophagy associated genes in the rat ADMSCs. Here, we demonstrated a novel data that have not been previously reported. Our results showed that DMI increases the proliferation of ADMSCs in a dose dependent manner under in vitro condition. Our experiment was performed using 125 μM and 250 μM of DMI, doses that have been shown to reduce LPS-induced inflammation in the other mammalian cells (19, 25). These doses had no inhibitory effect on the ADMSCs viability. Our qRT-PCR analysis revealed that treatment of ADMSCs with different doses DMI for 24 hours and 48 hours downregulated the transcription of *Fadd* and *Bax* genes and upregulated the transcription of *Bcl2* gene. Both doses of DMI had no obvious effect on the expression of *Caspase 8*, *Fasl* and *Caspase 3* genes. Here, we also demonstrated for the first time that DMI enhances the transcription level of autophagosome formation genes, including *Lc3b*, *Beclin1* and *P62*, while, it had no regulatory effect on transcription of *Atg5* and *Atg12* genes.

In accordance with our results, Lampropoulou et al. (17), has shown that treatment of bone marrow derived dendritic cells (BMDCs) with the DMI can protect them from hypoxia, an inducer cell death. A recent report by Muri et al have demonstrated that 4 octyl itaconate (4-OI) at low concentration protects the BMDCs against inflammation, while it promotes inflammatory apoptosis at high concentration (480 μM) (26). Functional study by Liu et al. (11) showed that 4-OI attenuated H_2O_2-induced neuronal cell death and apoptosis. Moreover, 4-OI treatment can reduce ischemia-reperfusion damage in the hepatocytes, which indicated an anti death effect of itaconate in the nonimmune cells (27). Taken together, these findings highlighted the protective effect of itaconate against cell death and apoptosis in various cells and this protection is depended on the cell type, dose of itaconate and physiological or pathological conditions of exposed cells.

Our results showed that DMI could upregulate the expression of autophagy associated genes, including *P62*, *Beclin1* and *Lc3b* concomitant with upregulation of the *Bcl2* as an anti-apoptosis gene. Despite the considerable advances in the biology of stem cells, understanding of the dual role of various immunometablites on the stem cell autophagy and apoptosis and their connections in the different physiological and pathological conditions remains incomplete. Understanding the actions of novel immunometabolites on the both of these pathways in the stem cells is an important area of research. It has been reported that autophagy, protection against stress condition, was induced by prolonged starvation, inflammatory agents and extrinsic death signals (28). Previous works have shown that *P62* can act as a main regulator of cell fate by controlling the autophagosomal degradation of several cellular proteins related to apoptosis and survival pathways (29). It has been reported that caspase-8 is degraded via autophagy pathway by interaction with p62 protein (30). BECLIN1 is another critical regulator of autophagy that directly interacts with anti-apoptotic BCL2 protein. Autophagy is induced by release of BECLIN1 from BCL2 by pro-apoptotic BH3 proteins (31). In addition, Caspase-3 inhibits the autophagy process by cleavage of BECLIN1 and production of an inactive, truncated form of BECLIN1 (32). In bone marrow MSCs, rapamycin induces autophagy markedly via reduction P62 accumulation and apoptosis, that suggests a protective role against apoptosis in the BMSCs for autophagy (11). Taken together we concluded that DMI by upregulation of *P62* and *Beclin1* may affect the components of the apoptosis machinery and enhance the cell viability and proliferation in the physiological condition.

Previous research indicated that ATG5 is required for autophagy induced vacuole formation by conjugation to human ATG12 homologue in a non-ubiquitin dependent pathway (9). It has been reported that ATG5 contributes to autophagic cell death by interacting with the FADD via Lys residue located in middle and C-terminal regions of ATG5. These data suggest that the induction of cell death by the ATG5 requires FADD as a downstream mediator (33). Although, the transcription of *Atg5* and *Atg12* was not altered in our study, down-regulation of *Fadd* transcription after exposure of ADMSCs to the DMI might result to enhance proliferation of ADMSCs following exposure to the DMI via inhibition of ATG5-ATG12 formation and an external pathway of apoptosis.

Our results showed that increasing of the cell proliferation potency of ADMSCs and upregulation of autophagy genes and anti-apoptotic genes after exposure to the DMI was accompanied by an increase in nuclear concentration of NRF2 protein. The NRF2 is a multifunctional and indispensable transcription factor that contributes to the autophagy and apoptosis of cells under stress condition via regulating the expression of several cytoprotective genes. Under normal conditions, Kelch-like ECH-associated protein 1 (KEAP1) binds to NRF2 in the cytoplasm, and act as an inhibitor of NRF2 activation. Different cellular stresses result in dissociation of KEAP1 from NRF2, consequently, *Nrf2* translocates to the nucleus where it activates the transcription of a host of cell defense genes (34). In accordance with our results, recent findings demonstrated that itaconate or its derivatives increases the alkylation of cysteine residues on the KEAP1, which enhances the degradation of KEAP1 and leads to translocation of NRF2 to the nucleus and its further activation of NRF2 and downstream gene transcription in the BMDCs and neuronal cells (11, 19, 23). Increased survival and proliferation along with altered expression of apoptotic genes and autophagy genes in the DMI treated

ADMSCs might be due to increased activation of NRF2 protein. To confirm this opinion several previous reports demonstrated that NRF2 overexpression improves MSCs survival under oxidative stress and protects MSCs against hypoxia induced apoptosis (35). According to our results, it is likely that DMI is involved in the transcription of apoptosis associated genes in the ADMSCs through activation of NRF2 pathway.

The relationship between NRF2 activation and autophagy has been indicated in previous reports and it was suggested that DMI might increase intranuclear NRF2 protein and cellular protection by affecting the expression of autophagy genes. To support this opinion, a recent study by Jiang et al. (36) has shown that P62 contributes to the activation of NRF2 by direct binding to KEAP1 and its autophagic degradation. This event induces KEAP1 degradation via autophagy and leaves NRF2 free to accumulate and translocate in the nucleus and facilitates the activation of NRF2 target genes. It has also reported that the suppression of NRF2 leads to autophagy and the osteoblastic differentiation of ASCs (37). These findings indicate that overexpression of *P62* in DMI treated ADMSCs in our study might contribute cell proliferation enhancement by activation of NRF2 and creating a positive feedback loop. Further researches are needed to confirm these mechanisms.

There are some limitations in this study that can be addressed in future research. The present study focused on determination of abundance of mRNA transcripts of apoptosis and autophagy associated genes and cell proliferation rate in the DMI treated ADMSCs. Application of specialized apoptosis and autophagy detection methods such as Annexin-V/propidium iodide method, Terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) assay, caspases activity assay and specific staining methods such as Hoechst 33258 and Acridine orange/ethidium bromide staining are suggested in future studies to better understanding of itaconate actions on stem cell fate and functions.

Conclusion

It has been demonstrated that MSCs gradually lose their proliferation and differentiation potential after long-term *ex vivo* culture. Our findings demonstrated that DMI by upregulation of some autophagy and anti-apoptosis associated genes and by activation of NRF2 may serve as a new cellular protective mechanism against stressful environment induced by exposure of stem cells to *ex vivo* condition. It is undeniable that the action of itaconate; as a novel immunometabolite, on MSCs is complex, and the relationship between itaconate and stem cell fate still requires further in vitro experiments. Knowing how itaconate acts on the complicated apoptosis and autophagy pathways may open the new research area for the development of novel protocols for culture and differentiation of MSCs in the *in vitro* condition.

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Authors' Contributions

All authors contributed to the study conception and design. F.S.; Performed the study and collected the data. M.R.T., D.D.; Designed the study, analyzed the results, drafted and revised the manuscript, critically for important intellectual content. All authors gave final approval of the version to be published.

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